Dealing with Transcriptional Outbursts during S Phase to Protect Genomic Integrity

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Abstract

Transcription during S phase needs to be spatially and temporally regulated to prevent collisions between the transcription and replication machineries. Cells have evolved a number of mechanisms to make both processes compatible under normal growth conditions. When conflict management fails, the head-on encounter between RNA and DNA polymerases results in genomic instability unless conflict resolution mechanisms are activated. Nevertheless, there are specific situations in which cells need to dramatically change their transcriptional landscape to adapt to environmental challenges. Signal transduction pathways, such as stress-activated protein kinases (SAPKs), serve to regulate gene expression in response to environmental insults. Prototypical members of SAPKs are the yeast Hog1 and mammalian p38. In response to stress, p38/Hog1 SAPKs control transcription and also regulate cell cycle progression. When yeast cells are stressed during S phase, Hog1 promotes gene induction and, remarkably, also delays replication by directly affecting early origin firing and fork progression. Therefore, by delaying replication, Hog1 plays a key role in preventing conflicts between RNA and DNA polymerases. In this review, we focus on the genomic determinants and mechanisms that make compatible transcription with replication during S phase to prevent genomic instability, especially in response to environmental changes.

Transcription during S Phase Is a Risk for Genomic Integrity

Replication and transcription are two essential processes that take place on the same template, the DNA, and coexist during S phase. The collision between the two machineries has deleterious consequences, and cells have evolved sophisticated mechanisms to avoid or resolve those conflicts to protect their genomic integrity.

S phase is the most vulnerable period of the cell cycle to accumulate DNA damage and genomic instability. First, the chromatin structure of the DNA is partially unwrapped to be more accessible to the DNA replication machinery; therefore, the resulting unprotected chromosomes are more susceptible to be damaged by both external and internal mutagenic agents that can directly modify the nucleotides. Second, the replication machinery must overcome several natural impediments such as tightly DNA bound proteins, unusual secondary structures or other enzymatic complexes that lead to replication fork stalling. Arrested replication forks are highly recombinogenic and prone to produce unscheduled chromosomal rearrangements [1,2]. One of the most relevant obstacles that the replisome must cope with is the transcription machinery. During S phase, transcription and replication coexist in space and in time, and therefore, they must be highly coordinated to avoid potential conflicts. It has been largely reported that collisions between replication and transcription machineries are detrimental for genomic integrity giving place to mutagenesis, DNA damage response, recombination events and genomic instability [3–8].

Cells Have Evolved Different Strategies to Prevent Conflicts between RNA and DNA Polymerases

Cells have developed a wide range of mechanisms to limit potential conflicts in order to guarantee the faithful DNA duplication and to protect genomic integrity in the presence of transcription (see Fig. 1).

Genomic organization in bacteria is crucial to avoid RNA and DNA polymerase conflicts

Bacterial genomes have evolved to minimize head-on collisions between replisome and transcription machinery [9–11]. Genomic organization shows a clear bias for genes encoded in the leading strand [11–13], providing co-directionality between replication and transcription machineries to avoid head-on

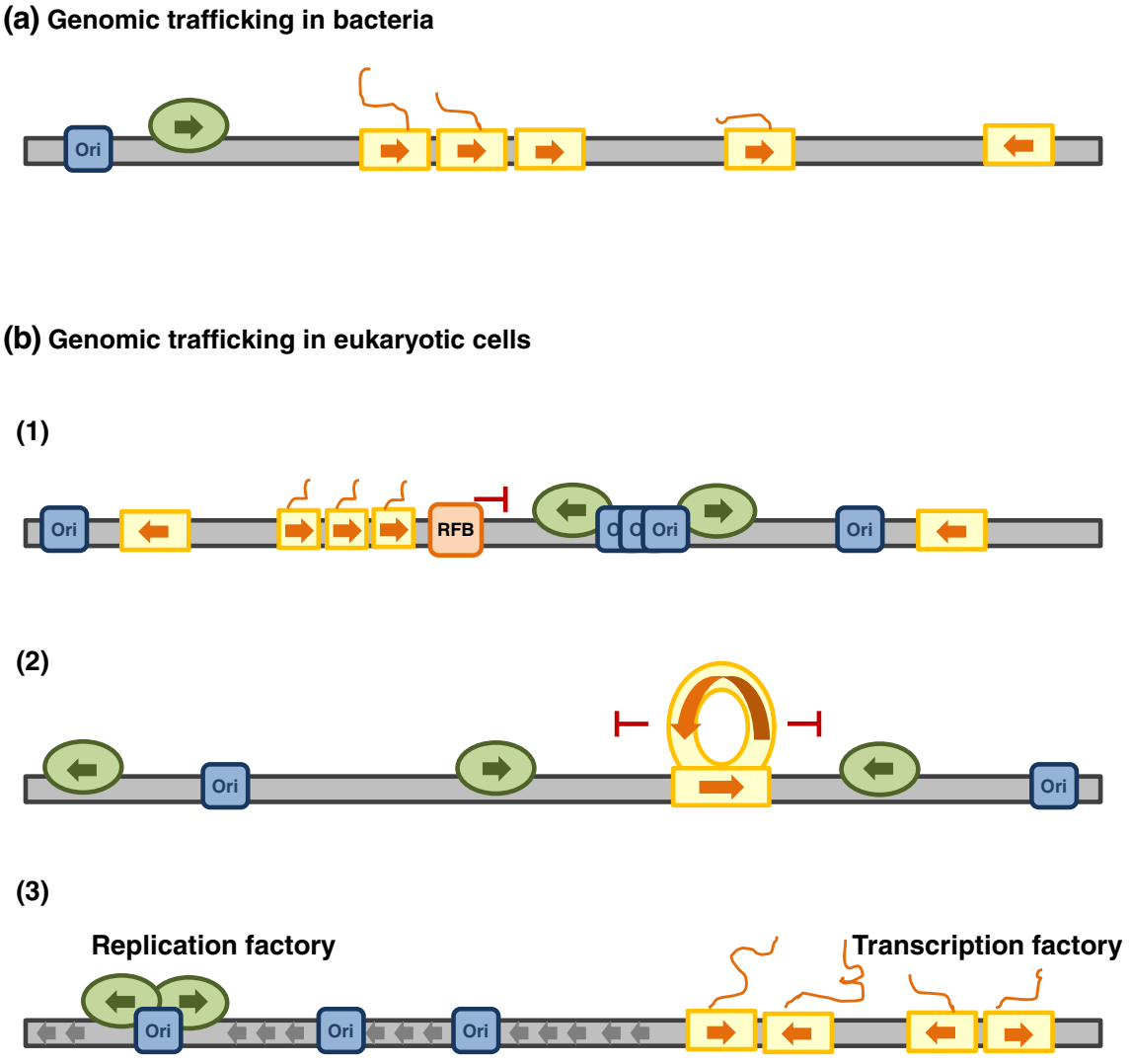

Fig. 1. Cells have evolved different strategies to prevent conflicts between RNA and DNA polymerases. (a) In bacteria, most of the genes, mainly core genes or highly transcribed genes, are encoded in the leading strand. This allows co-directionality with the replication forks avoiding head-on collisions and genomic instability. (b) Eukaryotic cells have developed several strategies to prevent head-on collisions between the transcription and replication machineries [1]. RFBs are highly specialized structures that prevent frontal encounters at regions with highly transcribed genes, like rDNA genes. RFBs are positioned at the downstream regions of each rRNA gene [2]. Gene looping insulates transcription and creates a barrier for the replication forks coming from both directions avoiding head-on and co-directional collisions [3]. Replication and transcription might take place in separated specialized regions in the nucleus keeping the two processes isolated.

clashes. For instance, in *Bacillus subtilis*, around 75% of the genes, which include most of the highly expressed genes, are located in the leading strand and replication proceeds normally with no detectable interference of the two processes [9,12,14].

In bacteria, cells suffer multiple co-directional encounters since the replication rate is about 15–20 times faster than transcription rate. However, except for the co-directionally positioned transcription terminators [15], they are normally benign or less harmful since the replisome can bypass transcription machinery by different mechanisms [10,16–18]. It is known that, upon co-directional encounters, the replication machinery can bump the RNA polymerase off the DNA template or it can bypass the RNA polymerase complex without blocking transcription [16,17,19,20].

In contrast, head-on orientation of replication and transcription has been shown to slow down the replication rate leading to genomic instability [18,21–23]. Moreover, head-on collisions impact fitness negatively by impeding the expression of highly transcribed genes [24,25] or by producing incomplete or mutated transcripts [26,27]. Correspondingly, the reversion of gene orientation of certain regions of DNA in bacteria has relevant deleterious consequences. In *B. subtilis*, most of the genes are transcribed from the leading strand. However, when certain genomic regions are reoriented, elongation rate of replication is clearly reduced by 50%. The severity of the replication block due to head-on collisions correlates with the transcription rate [9]. This is the case for the highly transcribed ribosomal RNA (rRNA) operons, which are always oriented away from the single bacterial origin of replication [12,28,29]. The artificial reorientation of the ribosomal genes in bacteria induces the SOS response, genomic instability and cell death [10]. All these studies supports that bacterial genomes have evolved to avoid head-on clashes and conserve co-directionality between replication forks and transcription bubbles [10,30].

Multiple mechanisms prevent RNA and DNA polymerases conflicts in eukaryotic cells

This simple but smart organization of bacteria genomes almost fulfills the necessity to coordinate replication and transcription to preserve genomic integrity. However, bacteria have only one replicon. In contrast, the eukaryotic genome is organized in multiple chromosomes, and DNA replication starts from multiple origins of replication distributed throughout them [31,32]. Thus, the mechanisms to negotiate the genomic trafficking are quite more complex. Conflicts between replication and transcription in the budding yeast *Saccharomyces cerevisiae* have been extensively studied over the last decade. Nowadays is well established that, as in bacteria, head-on collisions between both machineries in yeast cells results in a replication fork block and lead to transcription-associated recombination (TAR) and genomic instability [5,33,34]. Similarly, TAR is detected in mammalian cells only during S phase [35]. Correspondingly, transcription by all types of RNA polymerases induces fork arrest in *S. cerevisiae* only when transcribing in opposite sense to replication [6,36]. In addition to the establishment of R-loop structures by transcription depending on DNA sequences [37], it is well established that head-on collisions between RNA and DNA polymerases machineries in eukaryotic cells results in replication fork stalling and R-loop formation, causing DNA double-stranded breaks, hyperrecombination phenotypes, genomic instability and cell death [2,34,38–41]. Actually, there exists a type of fragile sites, the early replication fragile sites, where early origins are located at active genomic transcription regions [42]. These sites are hotspots for recombination caused by collisions between replication and transcription machineries that generate chromosomal rearrangements present in many types of cancer [42].

Despite the danger of the head-on collisions, it is not clear whether the collinear organization of replication and transcription present in bacteria is conserved in eukaryotic cells [43,44]. Instead, eukaryotic cells have developed alternative strategies to minimize the potential conflicts between transcription and replication. The best-characterized mechanism to prevent head-on clashes is through the polar replication fork barriers (RFBs) present at the rRNA gene arrays of yeast, mouse and human cells [45,46]. RFBs are natural pause sites defined by specific DNA sequences with tightly bound non-nucleosomal proteins that avoid the progression of the replication fork [47–50]. In *S. cerevisiae*, ribosomal DNA (rDNA) loci contain more than 150 copies of the 35S rRNA gene that are continuously transcribed by the RNA polymerase I throughout the cell cycle [51]. Replication through this rDNA region represents a real drawback for the replisomes travelling opposite to the transcription direction, in regions that, in addition, contain clusters of 3–5 active origins located downstream of these highly transcribed rDNA regions [52,53]. To coordinate both processes, yeast cells have developed a polar RFB downstream of the rDNA loci to arrest the replication forks that are progressing in the opposite direction of the RNA polymerase I [47,49]. Of note, albeit that role of RFB is widely accepted, it has been reported that deletion of the *FOB1* gene, which encodes a protein required for fork pausing at RFBs, did not increase the recombination rate at the rDNA loci [54]. Similar structures to prevent head-on clashes have been found at tRNA genes in *S. cerevisiae*. They are called replication fork pause sites, and as in rRNA genes, they serve to block replication forks that would otherwise move in the

opposite direction of the RNA polymerase III [6,55]. Therefore, despite the lack of the collinear organization of replication and transcription seen in bacteria, eukaryotic genomes preserve co-directionality by alternative mechanisms in those genomic areas with high levels of transcription. Highly transcribed RNA polymerase II-dependent genes also represent an impediment for replication fork progression [33], and there are experimental evidences suggesting that replication fork pausing at the RNA polymerase II-dependent occurs when head-on collisions take place [36]. It is worth noting that whereas the specific location in the genome of RNA polymerase I genes has allowed the evolution of specialized structures such as the RFB, RNA polymerase II-dependent genes are distributed throughout the chromosomes. Whether specialized structures or regulatory mechanisms coordinate conflicts between replication complex (RC) and RNA polymerase II is an intriguing question that is discussed below in this report.

The complexity of eukaryotic cells has allowed the development of alternative topological and architectural mechanisms to minimize the interference between replication and transcription. The spatial and temporal compartmentalization of chromatin at the nucleus allows replication and transcription-related processes to occur within spatially and temporally separated domains [56,57]. It has been reported that the presence of factories in specialized locations permits that DNA and RNA polymerases do not travel along the chromatin but rather work at restricted regions in which the DNA is pulled [58,59]. This network-like organization would keep these domains separated throughout S phase, and only 3% of the transcription foci would overlap with transcription sites in early S phase [56]. On the other hand, some transcription strategies such as DNA looping also contribute to topologically separate transcription and replication. Apart from increasing the capability of mRNA transcription, gene looping insulates transcription and generates a barrier for incoming replication forks independently of the direction of the replication [7].

Exceptional Activation of Transcription during S Phase Requires Dedicated Mechanisms to Prevent RNA and DNA Polymerase Collisions

The large number of strategies that prokaryotic and eukaryotic organisms have developed to minimize collisions between transcription and replication highlights the relevance of coordinating genomic trafficking to preserve genomic integrity. However, clashes between transcription and replication machineries still occur in eukaryotic cells, for instance, when transcription occurs in replicating cells, having relevant implications for cancer, or when there is an outburst of transcription during S phase [10,60].

Environmental stresses induce rapid transcriptional outbursts

Cells are exposed to constant changes in the environment. Exposure to environmental conditions such as increases in oxidative stress, changes in external pH, nutrient supply, temperature changes or imbalances in osmolarity require changes in the intracellular physiology to maximize cell survival under the new environmental conditions. Eukaryotic cells have evolved sophisticated sensing mechanisms and signal transduction systems that can produce accurate dynamic outcomes in response to environmental changes. Those signal transduction systems control almost any aspect of cell physiology and, in general, a major outcome in response to environmental changes consists in important changes in gene expression [61–63].

The p38/Hog1 SAPKs play a key role upon stress

The response of cells to osmostress has been widely studied. Osmostress rapidly induces the activation of a family of signaling kinases known as stress-activated protein kinases (SAPKs). In budding yeast, the HOG (*h*igh-*o*smolarity *g*lycerol) pathway is the main mediator of cellular adaptation upon osmostress and it is one of the best-characterized SAPK cascades in eukaryotes (revised in Refs. [64–68]). The HOG signaling system consists of two upstream independent branches, with their respective osmosensors, that converge on the Pbs2 MAPKK and the Hog1 SAPK [68,69]. Upon stress, the Hog1 SAPK is rapidly and transiently activated and there are specific mechanisms that are designed for such a rapid activation [70–72]. The activation of Hog1 correlates very well with its accumulation into the nucleus [73,74].

In mammalian cells, both the architecture and the main players of the pathway are highly conserved. The p38 SAPK is the mammalian homologue of the yeast Hog1 SAPK [68,75,76]. In contrast to Hog1, which is mainly activated upon osmostress, p38 is activated by a multitude of external stimuli such as cytokines, DNA damage, oxidative and heat stresses as well as osmostress. The central core of the p38 pathway in mammals is similar to the HOG in yeast, albeit that the molecular activation mechanisms leading to its activation to stress are not well defined. Moreover, in contrast to Hog1, p38 function not only is crucial for the acute response to cellular insults but also plays key roles in controlling differentiation, proliferation, apoptosis, cell morphology and immune response [77,78]. In response to osmostress, Hog1/p38 activation elicit the program for cell adaptation

required for cell survival including cell metabolism, protein translation, cell cycle progression and the control of gene expression [64–67].

p38/Hog1 orchestrates gene expression upon stress

In general, stress causes a general down-regulation of gene expression combined with the induction of a specific set of stress-responsive genes, resulting in a change in the gene expression landscape of the cell [79]. Both in mammals and in yeast, the p38-related SAPKs are important regulators of transcription upon stress. Analyses of the transcriptional changes mediated by Hog1 in response to osmostress have shown that cells rapidly and efficiently adjust a full transcriptional program in response to extracellular stimuli. Expression profiling studies have shown that about ~300 to ~600 genes are regulated upon stress [79–87]. A similar extent of genes with induced transcription has been reported in mammals by p38 upon osmostress [88].

The p38-related Hog1 SAPK is the master protein for reprogramming gene expression in response to osmostress through different specific transcription factors [79,83,85]. Hog1 is recruited to the osmoresponsive genes by these specific factors [89–97]. Once bound to chromatin, Hog1 serves as a platform to recruit RNA polymerase II [79,92] and associated transcription factors such as SAGA, Mediator and the histone deacetylase Rpd3 [91,98–100]. Hog1 is present also at the coding regions of stress-responsive genes [79,94–96], where its kinase activity is essential for increased association of RNA polymerase II and efficient mRNA production in response to osmostress [79,96,101,102]. Moreover, nucleosome positioning of specific stress-responsive loci is altered dramatically in a Hog1-dependent manner [79] and the modification to chromatin is mostly regulated by the interplay of the INO80 and the RSC chromatin remodeling complexes [103,104]. The chromatin dynamics set a threshold for gene induction upon Hog1 activation [105]. In addition to its impact in gene induction, the Hog1 SAPK controls mRNA stability [84,86,87], mRNA export by targeting specific nucleoporins in the NPC [106] and mRNA translation [107]. Thus, the Hog1 SAPK plays a key role in the regulation of mRNA biogenesis by controlling several steps in the transcription process [61,65,108,109]. Remarkably, the change on the transcriptional capacity of the cells upon stress also occurs in replicating cells [110]. Thus, it is conceivable that cells must deal with the possibility that a significant amount of transcription might coincide with the initiating or ongoing replication that might lead to TAR (Transcription Associated Recombination) [40,111,112]. Hence, these two major dynamic complexes, replication and transcription, could feasibly interfere with each other. Indeed, there are over 300–600 genes transcriptionally induced by Hog1 and approximately 400 origins of replication [79,113]. Thus, a transient block of DNA replication in response to osmostress might be important to avoid the collision of the two essential machineries, which is known to lead to genomic instability [40,111,112].

Hog1 regulates S phase upon osmostress

It has been known for a long time that environmental stresses lead to a transient cell cycle arrest and that the bypass of this cell cycle delay is detrimental for cell survival [114–119]. Thus, cells activate checkpoint surveillance mechanisms in response to extracellular stimuli to modulate cell cycle progression and to permit adaptation to change environmental conditions. The Hog1 and p38 SAPKs regulate multiple stages of the cell cycle by acting on core components of the cell cycle machinery [78,120–122]. For instance, Hog1 controls G1/S transition by the down-regulation of cyclin expression and the stabilization of the Sic1 cyclin-dependent kinase inhibitor (CDKi) [123,124]. Similarly, p38 down-regulates cyclin expression and phosphorylates the p57 CDKi during G1 in response to osmostress [125,126]. Cells unable of delaying cell cycle progression upon osmostress, both in yeast and in mammals, display reduced viability upon those conditions [123,126]. Thus, regulation of cell cycle progression is critical to maximize cell survival upon stress. Of note, the Hog1 and p38 SAPKs are important not only to regulate the G1/S transition but also to regulate other phases of the cell cycle such as G2/M in response to stress [120,127], suggesting that, in the presence of stress, cells need to delay cell cycle to permit the generation of adaptive responses before progressing into the next phase of the cell cycle.

Remarkably, the Hog1 SAPK also plays a crucial role once the cells are already in S phase [110,128]. It is known for a long time that, in response to DNA damage or under replication stress, cells activate the Rad53-dependent S-phase checkpoint to cope with multiple genotoxic agents that endanger the proper progression and completion of DNA replication [129–131]. In response to osmostress, the Hog1 SAPK delays S-phase progression independently of this S-phase checkpoint [110,128], indicating that an additional checkpoint to respond to environmental insults during S phase is required for adaptation to osmostress [128].

Hog1-Mrc1 blocks S phase to protect genomic integrity upon osmostress

Activation of the Hog1 SAPK occurs within minutes, which correlates with the nuclear accumulation of the SAPK and its association to chromatin (see above). With similar kinetics, the Mrc1 protein, a component of RC, is phosphorylated by Hog1 upon stress. Mrc1 plays a critical role both in normal conditions and under replication stress. During a normal S phase, Mrc1 links the helicase and the polymerase to permit DNA

replication to proceed properly [132]. In the presence of DNA damage or under replication stress, Mrc1 has an essential function as a mediator in the checkpoint response, when after phosphorylation by Mec1, and allows the phosphorylation of the effector checkpoint kinase Rad53 [133]. Of note, phosphorylation of Mrc1 by the SAPK occurs in different sites to those targeted by Mec1 upon DNA damage, pointing out Mrc1 as a signal integrator regulated by different kinases that respond to internal and external insults [128]. Hog1 phosphorylation of Mrc1 delays DNA replication by a dual mechanism, inhibiting origin firing and slowing down RC progression [128]. Cells carrying a non-phosphorylatable allele of *MRC1* (*mrc1*^{3A}) by Hog1 do not delay replication upon stress and display a dramatic increase in TAR, genomic instability and Rad52 foci [128]. Therefore, upon stress, when the cell needs to activate transcription, the transient block of DNA replication is critical to maintain genomic integrity and to maximize cell survival (see Fig. 2).

The Need of a Dedicated S-Phase Checkpoint To Cope with Massive Transcriptional Changes Caused by External Environmental Insults

Why cells need to block S phase progression upon osmostress is a key question. In response to osmostress, Hog1 orchestrates a fast and transient activation of transcription of hundreds of stress-responsive genes that also occurs during S phase. Induction of gene expression might represent an important drawback in replicating cells since the risk of collision between the replication and the transcription machineries significantly increases [36]. Remarkably, in a TAR assay, in which the induction of transcription was mediated by Hog1, cells carrying the *mrc1*^{3A} allele are unable to delay replication upon stress and displayed a dramatic increase in TAR only when the stress-responsive promoter was induced [128]. Therefore, the delay on replication might serve to coordinate the stress-induced transcription with DNA replication to protect genomic integrity. Here, the SAPK plays a dual role during stress response; it orchestrates a massive transcriptional response required for proper adaptation and it slows down the replication process, thus avoiding conflicts between transcription and replication machineries.

The mechanisms to prevent genomic instability in response to massive changes of transcription in response to osmostress highlight the necessity of cells of coordination with DNA replication. Therefore, it could be that other stresses inducing massive changes in gene expression such as heat or oxidative insults also represent a real challenge for replicating cells. Therefore, cells might have similar checkpoint pathways to delay S phase and protect genomic integrity in response to different environmental insults. Whether

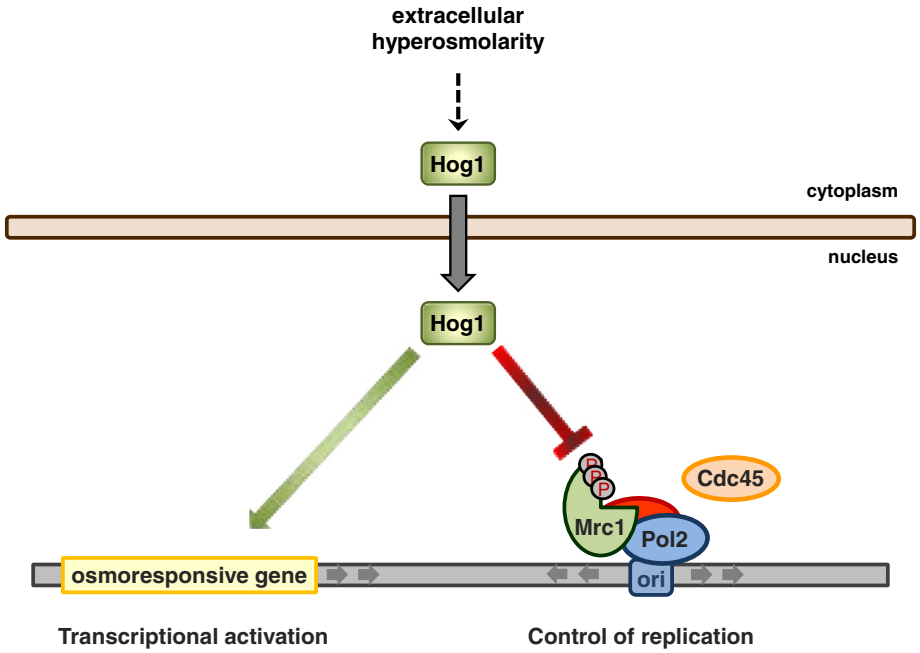

Fig. 2. Schematic diagram of the role of Hog1 upon stress in coordinating transcription with DNA replication. Upon osmostress, the activated Hog1 SAPK activates transcription and blocks DNA replication by phosphorylating three specific sites in Mrc1, a protein of the RC. The phosphorylation of Mrc1 by Hog1 changes its affinity toward DNA Pol2 and delays Cdc45 loading that prevents origin firing. Thus, Hog1 coordinates the process of gene expression and DNA replication in response to osmostress to prevent collisions between transcription and replication machineries to protect genomic integrity.

Mrc1 is also the key molecule to delay S phase upon other environmental stresses is an interesting question that remains to be addressed. Alternatively, other means to delay DNA replication might exist to protect genomic integrity upon environmental stimuli.

Remarkably, it has been recently shown that genomic instability *per se* elicits a massive transcriptional response that resembles to that of an environmental stress response [134]. It was reported that, independently of the type of chromosome aberration, aneuploid cells of yeast, plants, mice and human organisms induced similar changes in the gene expression pattern. These cells show a common transcriptional signature called ESR (*e*nvironmental *s*tress *r*esponse) [80] that involves a down-regulation of genes related with cell fitness and a massive up-regulation of the stress-responsive genes [134]. This opens a new scenario in which the coordination of replication and transcription must be essential to avoid collisions between both machineries, avoiding that aneuploid cells accumulate higher levels of genomic instability. If that mechanism exists, it would be crucial at early steps of tumorogenesis to avoid the exponential accumulation of genomic instability that normally leads to tumor formation.

Conclusions and Perspectives

Protecting genomic integrity is essential to guarantee the faithful transmission of genetic information through cell generations. This is highlighted by the presence of multiple surveillance mechanisms to safeguard the genome at all phases of the cell cycle. Among them, the most relevant is the DNA damage checkpoint pathway that operates in S phase protecting the replication of the DNA from all kind of genotoxic agents. However, while replicating, cells must cope with other drawbacks that alters the normal process of replication such as the presence of RNA polymerases associated to DNA. Prokaryotic cells seem to have chosen strategies that involve genomic organization in which they have placed highly expressed genes in the leading strand and, thus, preventing the head-on collision between DNA and RNA polymerases. The presence of additional elements or the spatial and temporal coordination of the two processes has also been exploited in eukaryotic cells to minimize the risk of collision.

However, there are special circumstances in which cells in S phase are subjected to major changes in their transcriptional capacity. For instance, in response to environmental stresses, cells dramatically change their gene expression pattern to permit the adaptation to the new external conditions to maximize cell survival. This transcriptional outburst dramatically increases the risk of collision between the replication and transcription machineries, challenging genomic integrity. To coordinate both processes, cells have evolved a dedicated checkpoint mechanism that delays S-phase progression while permitting the proper transcription of stress-responsive genes. Upon osmostress, the Hog1 SAPK phosphorylates Mrc1, a protein of the RC, to block S phase to avoid conflicts between replication and transcription machineries. Interestingly, the novel Hog1-Mrc1 checkpoint pathway works independently of the DNA damage checkpoint pathway. Therefore, cells have evolved two independent pathways to protect DNA replication from internal and external insults, highlighting the relevance of protecting genomic integrity whatever the nature of the insult is. It will be interesting to explore whether similar mechanisms to negotiate genomic trafficking operate in other stress situations that involve an outburst of transcription.

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SAPK, stress-activated protein kinase; rRNA, ribosomal RNA; TAR, transcription-associated recombination; RFB, replication fork barrier; rDNA, ribosomal DNA; RC, replication complex.

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